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The $G\alpha_q$ and $G\alpha_{11}$ Proteins Couple the Thyrotropin-releasing Hormone Receptor to Phospholipase C in GH_3 Rat Pituitary Cells*

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Thyrotropin-releasing hormone stimulates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) in GH_3 cell membranes. The stimulation of the phosphoinositide phospholipase C (PI/PLC) activity can be blocked by incubation of GH_3 membranes with polyclonal antibodies directed against a peptide derived from the C-terminal region of Gαq and Gα11. Antibodies directed against the C-terminal region of other Gα-subunits had no detectable effect. The inhibition was specific since addition of the peptide that was used to prepare the antibody completely reversed the inhibition. Further evidence for the coupling of the TRH receptor to $G\alpha_q$ or $G\alpha_{11}$ comes from a reconstitution experiment in which human embryonic kidney cells were transiently transfected with cDNAs corresponding to the TRH receptor, $G\alpha_q$ or $G\alpha_{11}$. The PIP_2 hydrolysis detected with membranes from cells that over-expressed the TRH receptor alone was low, however, co-expression with the $G\alpha_q$ or $G\alpha_{11}$ subunits produced a synergistic stimulation of PI-PLC activity. In contrast, co-expression of these α-subunits with the M2 muscarinic acetylcholine receptor induced a weak stimulation of PIP_2 hydrolysis. The results presented here suggest that the TRH-dependent stimulation of PI-PLC in GH_3 cells is mediated through the G-protein α-subunits, $G\alpha_q$ and/or $G\alpha_{11}$.

Thyrotropin-releasing hormone (TRH)[1] regulates prolactin secretion in anterior pituitary cells. GH_3 and GH_4 rat pituitary tumor cells (Tashjian *et al.*, 1968; Tashjian, 1979) posses high affinity membrane receptors for the peptide and secrete prolactin following stimulation with TRH. A large body of data supports the idea that when intact cells are exposed to TRH there is an activation of phosphatidylinositol 4,5-bisphosphate hydrolysis by a phospholipase C leading, within seconds, to an increase in inositol triphosphates and 1,2-diacylglycerol, rapidly followed by an elevation of cytoplasmic calcium concentration and protein phosphorylation (reviewed in Gershengorn, 1986). These effects are thought to be important in stimulation of prolactin release and synthesis in GH_3 cells.

A great deal of evidence supports the notion that a heterotrimeric G-protein couples the TRH receptor to the phosphoinositide pathway: (i) TRH activates a high affinity GTPase in GH_3 cells membranes (Hinkle and Philips, 1984); (ii) addition of GTP converts the membrane receptor to a lower affinity state (Hinkle and Kinsella, 1984); (iii) TRH and GTP or GTPγS stimulate the accumulation of inositol polyphosphates in a time- and concentration-dependent manner (Straub and Gershengorn, 1986; Lucas *et al.*, 1985; Aub *et al.*, 1986, 1987); (iv) the TRH receptor is a member of the large family of receptors that posses seven membrane-spanning domains and share the characteristic topological structure of a G-protein-coupled receptor (Straub *et al.*, 1991). Although there is evidence supporting the involvement of a G-protein, it is not clear which member of the G-protein family couples the TRH receptor to the activation of a phospholipase C leading to the increase in the levels of inositol triphosphates. *Bordetela pertussis* toxin (PTX) has been widely used to demonstrate the coupling of different receptors to G-proteins (Ui, 1990). The toxin uncouples G-protein function from receptor activation by catalyzing the ADP-ribosylation of most of the members of the G_i class α-subunits. Pretreatment of the GH_3 cells with pertussis toxin fails to affect TRH receptor affinity or to inhibit the TRH stimulation of inositol triphosphate formation (Aub *et al.*, 1986, 1987; Martin *et al.*, 1985), suggesting that a PTX-resistant Gα subunit couples the TRH receptor to PLC activation.

Based on the amino acid sequence of the α-subunits it is possible to predict which α-subunits are targets for PTX-mediated ADP-ribosylation. The G_q class including $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, and $G\alpha_{15}/G\alpha_{16}$ (Pang and Sternweis, 1990; Strathmann and Simon, 1990; Nakamura *et al.*, 1991; Wilkie *et al.*, 1991; Amatruda *et al.*, 1991), lacks the cysteine residue four amino acids from the C terminus which is the target for PTX modification. Gα-subunits in the G_{12} (Strathmann and Simon, 1991; Nakamura *et al.*, 1991) and G_s classes, in addition to $G\alpha_z$, also lack this critical cysteine residue but only G_q class α-subunits have been implicated in PTX-resistant coupling to phospholipase C activation. A 42-kDa G-protein from rat brain (Pang and Sternweis, 1990) and liver membranes (Taylor *et al.*, 1990) with amino acid sequence identity to $G\alpha_q$ and $G\alpha_{11}$ activated the β1 isotype of phospholipase C but not the γ1 or δ1 isotypes (Smrcka *et al.*, 1991; Taylor *et al.*, 1991). Additional evidence supporting the involvement of $G\alpha_q$ and $G\alpha_{11}$ proteins in PLC activation was provided by transiently transfecting cells with $G\alpha_q$ and $G\alpha_{11}$ cDNAs. Membranes derived from these cells show that both $G\alpha_q$ and $G\alpha_{11}$ stimulate β1 isoform of PLC (Wu *et al.*, 1992). Finally, the involvement of members of the G_q class α-subunits in ligand-mediated stimulation of PLC activity by vasopressin, angiotensin, bradykinin, and thromboxane A_2 receptors has recently been demonstrated (Gutowsky *et al.*, 1991; Shenker *et al.*, 1991).

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[1] The abbreviations used are: TRH, thryrotropin-releasing hormone; G-protein, guanine nucleotide-binding regulatory protein; $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ etc., the α-subunit subtype of G-protein; PI-PLC, phosphoinositide-specific phospholipase C; PTX, pertussis toxin; GTPγS, guanosine 5′-*O*-3′-thiotriphosphate; GDPβS, guanosine 5′-*O*-2′-thiodiphosphate; PIP_2, phosphotidylinositol 4,5-bisphosphate.

In this paper we show that GH_3 cells express proteins that react with antibodies specific to $G\alpha_q$ and/or $G\alpha_{11}$. We have also used antibodies directed against a region common to $G\alpha_q$ and $G\alpha_{11}$ to demonstrate the involvement of these G-proteins in the TRH activation of phosphoinositide hydrolysis in membranes of GH_3 cells. Furthermore, we have demonstrated that in HEK-293 cells transiently transfected with the TRH receptor cDNA alone or co-transfected with $G\alpha_q$ or $G\alpha_{11}$ cDNAs the TRH receptor couples to $G\alpha_q$ or $G\alpha_{11}$ leading to the activation of the $\beta 1$ isoform of the phosphoinositide phospholipase C. This constitutes additional evidence for a role of the G_q class in coupling ligand-bound receptors to activation of phospholipid metabolism.

EXPERIMENTAL PROCEDURES

Materials

Guanosine-5′-*O*-(3′-thiotriphosphate) and guanosine-5′-*O*-(2′-thiodiphosphate) were purchased from Calbiochem. L-α-Phosphatidyl-D-*myo*-inositol-4,5-bisphosphate was obtained from Boehringer Manheimm. [^{3}H]L-α-Phosphatidyl-D-*myo*-inositol-4,5-bisphosphate was purchased from Du Pont-New England Nuclear. L-α-Phosphatidylserine and L-α-phosphatidylethanolamine were from Avanti Polar-lipids, Inc. Thyrotropin-releasing hormone and carbachol were obtained from Sigma. Cell culture reagents were from GIBCO.

Methods

Cell Culture and Transfections— GH_3 cells were grown as described previously (Tashjian *et al.*, 1968). Cells were maintained at a density of 10^5-10^6 cells/ml in flasks containing Ham's F-10 medium supplemented with 15% horse serum and 2.5% bovine serum in a humidified atmosphere of 5% CO_2. Cells were plated 3 days before preparation of membranes onto polylysine-coated plastic dishes. HEK-293 human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. 2×10^6 cells/10-cm dish, seeded a day before, were transiently transfected with 10 μg of cytomegalovirus expression vectors by the calcium phosphate precipitation method (Graham and Van der Eb, 1973). The cDNAs corresponding to $G\alpha_{11}$ and $G\alpha_q$ were cloned into pCIS (Gorman *et al.*, 1990) and the TRH receptor cDNA was in the pcDNA-I vector (Invitrogen). The cells were exposed to the precipitate for 14 h at which time the medium was changed, and 34 h later the cells were harvested.

Preparation of Membranes—Cell monolayers of GH_3 cells were rinsed twice with phosphate-buffered saline and scraped into 5 ml of homogenizing buffer (50 mM HEPES, pH 7.0, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.5 mg/ml leupeptin, and 0.5 mg/ml aprotinin). Cells were allowed to swell for 10 min on ice and then were lysed by 10 strokes of a Dounce glass homogenizer. Cell homogenates were cleared by centrifugation at 500 × *g* for 5 min. The supernatant was then spun down at 100,000 × *g* for 40 min. Membrane pellets were resuspended in homogenizing buffer at a concentration of 2–3 mg/ml, and aliquots were frozen in liquid nitrogen for storage at −70 °C until use. Protein concentration was determined by the Bradford method (Bio-Rad). HEK-293 cell membranes were prepared as above with the following modifications: after centrifugation, membranes were resuspended in 0.8–1 ml of cold extraction buffer (1:1 mixture of 2 M KCl and homogenization buffer), followed by rocking at 4 °C for 2 h in order to wash off the endogenous PI-PLC enzyme. The membrane suspensions were spun down at 100,000 × *g* for 40 min, and the pellets were resuspended in 3 ml of homogenation buffer, followed by centrifugation at 100,000 × *g* for 40 min. The final pellets were resuspended in 400 μl of homogenization buffer, and aliquots were frozen in liquid nitrogen for storage at −70 °C until use.

Antibody Purification—Antibodies were made in rabbits by using synthetic peptides (described in Fig. 1) conjugated to keyhole limpet hemocyanine. The antibodies were purified by affinity chromatography using the synthetic peptides coupled to Affi-Gel 15 (Bio-Rad). Elution was performed with 3 M NaSCN containing 0.1% bovine serum albumin. The eluants were dialyzed against 20 mM phosphate buffer, aliquoted, and stored at −20 °C until use.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot—SDS-polyacrylamide gel electrophoresis was performed as described (Laemmli, 1970). Membranes prepared as described above were resuspended in SDS loading buffer, boiled for 5 min, centrifuged, and loaded onto a 12% polyacrylamide gel. Gels were electrotransferred to nitrocellulose membranes and immunostained with antibodies.

PIP_2 Hydrolysis Assay—Phospholipid vesicles were prepared as described (Hofmann and Majerus, 1982). Assays were done essentially as described (Wu *et al.*, 1992). The indicated amount of membranes in 50 mM HEPES, pH 7.0, 0.2 mM EDTA, 1 mM dithiothreitol, were added to 30 μl of assay buffer (50 mM HEPES, pH 7.0, 100 mM KCl, 6 mM $MgCl_2$, 0.6 mM $CaCl_2$, and 2 mM EGTA) plus 10 μl of PIP_2 (10,000–12,000 cpm of [^{3}H]PIP_2) and incubated for 10 min on ice. For the antibody inhibition assay membranes were preincubated for 2 h with 10 μl of antibody, at concentrations indicated, prior to the addition to the reaction mixture. For the competition assay with peptide (no. 736), 10 μl of 5 μM CT76 antibody was mixed with 2 μl of serially diluted peptide (at the concentrations indicated) before addition to the membranes and incubated for 2 h. The reaction was started with the addition of GTPγS with or without TRH followed by incubation at 37 °C for 15 min. The reaction was stopped by the addition of 0.5 ml of chloroform/methanol/HCl (100:100:0.6) with 0.15 ml of 1 N HCl containing 5 mM EGTA. Phases were separated by centrifugation, and 200-μl aliquot of the upper aqueous phase was taken for liquid scintillation counting. For the reconstitution experiment with HEK-293 cells, 5 μg of membranes depleted of endogenous PI-PLC but supplemented with purified bovine brain PI-PLC β1 (Ryu *et al.*, 1987) were used in the PIP_2 hydrolysis assay.

RESULTS

Characterization of Gα-subunits of the G_q Class in GH_3 Cell—We used different affinity purified antipeptide polyclonal antibodies to investigate the presence of the α-subunits of the G_q family in membranes of the rat pituitary cell line (GH_3). Fig. 1 shows the sequences of the peptides used to prepare the different antibodies.

Since the C-terminal sequences of $G\alpha_q$ and $G\alpha_{11}$ (Strathmann and Simon, 1990) proteins are identical and differ by only two amino acids from $G\alpha_{14}$ (Wilkie *et al.*, 1991), we predicted that antibodies against the C-terminal peptides would react with the three α-subunits. As shown in Fig. 2, two affinity purified antibodies, CT76 and CT90, detected a 42-kDa protein in Western blots of crude membranes prepared from HEK-293 cells transiently transfected with either $G\alpha_q$, $G\alpha_{11}$, or $G\alpha_{14}$ cDNAs. Importantly, the CT90 antibody showed a strong reactivity with $G\alpha_q$ protein. Membranes from HEK-293-untransfected cells showed very weak antibody reactivity. Both antibodies detected a band of 42 kDa in membranes of GH_3 cells.

The levels of expression of each α-subunit in GH_3 cells were assessed using internal specific antibodies made against peptide sequences unique for each α-subunit (Fig. 1). The specificity of the antibodies was verified by analyzing crude membranes of HEK-293 cells transiently transfected with the cDNAs corresponding to each of the Gα-subunits (Fig. 2). Western immunoblots of GH_3 membranes show a 42-kDa protein that strongly reacts with the $G\alpha_q$-specific antibody (CT38) and also with the $G\alpha_{11}$-specific antibody (CT31). The $G\alpha_{14}$-specific antibody (CT92) showed no apparent reaction

Ab	Gα	Amino Acid Sequence	Position	Peptide
CT76	Gαq-11	QLNLKEYNLV	C-Terminus	(#736)
CT90	Gα14	QLNLREFNLV	C-Terminus	(#889)
CT56	Gα15-16	RDSVLARYLDEINLL	C-Terminus	(#718)
CT38	Gαq	KVSAFENPYVDAIKS	Internal (119-133)	(#620)
CT31	Gα11	KVTTFEHQYVNAIKT	Internal (119-133)	(#625)
CT92	Gα14	DKVTALSRDQVAAIKQL	Inetrnal (118-134)	(#894)

FIG. 1. **Antibody description.** Gα-specific antibodies were raised against the different peptides shown in the figure. The peptide position in the G-protein is indicated.

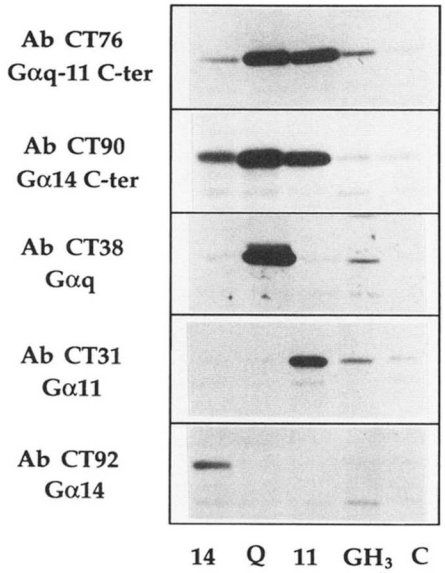

FIG. 2. **Expression of the G_q subfamily α-subunits in GH_3 and HEK-293 membranes.** Membranes from GH_3 and HEK-293 transiently transfected cells with $G\alpha_{14}$ (*14*), $G\alpha_q$ (*Q*), and $G\alpha_{11}$ (*11*) cDNAs and pCIS empty vector (*C*) were analyzed by SDS-PAGE and immunoblotting with: *CT76*, $G\alpha_q$-11 C-terminal antibody; *CT90*, $G\alpha_{14}$ C-terminal antibody; *CT38*, $G\alpha_q$-specific antibody; *CT31*, $G\alpha_{11}$-specific antibody; and *CT92*, $G\alpha_{14}$-specific antibody.

with GH_3 membranes. We conclude that GH_3 cells express $G\alpha_q$ and $G\alpha_{11}$ subunits. However, under the conditions used in our assay we could not detect $G\alpha_{14}$. Other members of the G_q subfamily are $G\alpha_{15/16}$, but their common C-terminal sequence differs significantly from that of the rest of α-subunits (Amatruda *et al.*, 1991; Wilkie *et al.*, 1992). Affinity purified antibody against C-terminal $G\alpha_{15/16}$ peptide (CT56) did not recognize any protein in GH_3 membranes (data not shown).

Antibody Inhibition of TRH-induced PIP_2 Hydrolysis—We used a cell-free membrane system in order to test the ability of specific antibodies to block TRH-induced phospholipase C activity. GH_3 membranes containing endogenous $G\alpha$-subunits, PI-PLC, and TRH receptor were mixed with phospholipid vesicles containing radioactive substrate ($[^3H]PIP_2$). Fig. 3*A* shows the dose-response effect of TRH. In the presence of GTPγS, TRH stimulated the hydrolysis of phosphatidylinositol 4,5-bisphosphate in GH_3 membrane suspensions. Half-maximal stimulation by TRH occurred at 30 ± 10 nM. The concentration dependence of the TRH effect is consistent with TRH binding to its receptor in the membrane, as shown previously (Hinkle and Tashjian, 1973; Straub and Gershengorn, 1986).

The dose-response effect of GTPγS in the absence and presence of the ligand is shown in Fig. 3*B*. At concentrations at or below 1 μM, the effect of GTPγS alone was negligible, but higher concentrations of GTPγS produced an increase in the PIP_2 hydrolysis. We chose 1 μM GTPγS as the optimal condition to detect the ligand-dependent response of receptor activation. At this concentration TRH showed a clear concentration-dependent activation of PLC. We also tested the addition of GDPβS. GDPβS is able to suppress agonist-independent activation of the G-protein in receptor-induced PLC activation in membrane preparations (Gutowsky *et al.*, 1991). We found that addition of GDPβS markedly decreased TRH-independent PIP_2 hydrolysis but also substantially decreased the TRH-dependent response (data not shown).

To determine whether $G\alpha_q$ and $G\alpha_{11}$ couple the TRH receptor to PLC activation, we incubated GH_3 membranes with antibodies prior to stimulation with the nucleotide analog and

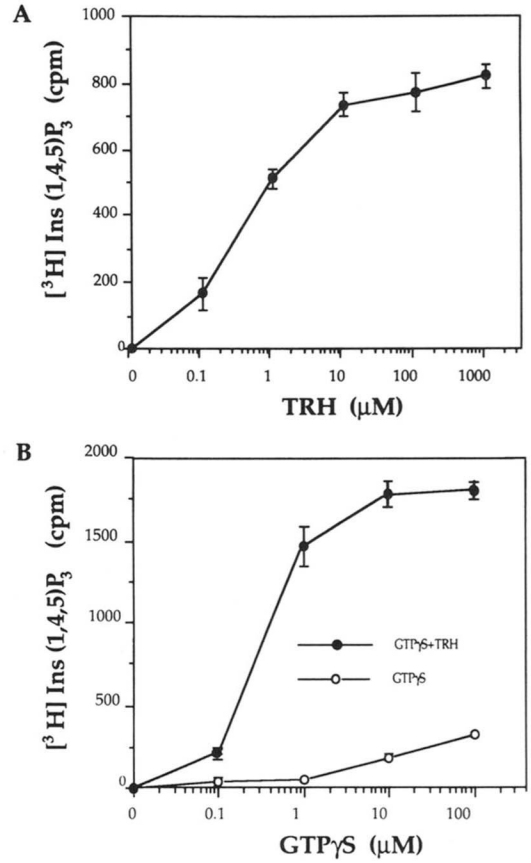

FIG. 3. **Activation of PIP_2 hydrolysis by TRH in GH_3 membranes.** *A*, dependence on TRH concentration. 6 μg of membrane suspension were incubated in presence of 1 μM GTPγS and different concentrations of TRH as indicated. *B*, dependence on GTPγS concentration. 6 μg of membrane suspension were incubated in the presence of different concentrations of GTPγS as indicated and in the presence (●) or absence (○) of 100 μM TRH. PIP_2 hydrolysis was performed as described under "Experimental Procedures." The level of PIP_2 hydrolysis in control incubations (without membranes but with TRH and GTPγS) were subtracted from all values. Data shown represent the mean value of duplicate determinations. *Vertical bars* denote S.E.

the hormone. Incubation of membranes with affinity purified $G\alpha_q/G\alpha_{11}$ common C-terminal antibody (CT76, 1.25 μM) reduced the TRH induction of PIP_2-hydrolysis by 75% (Fig. 4). Inhibition by antibody CT90 was even more pronounced (90%). Both antibodies also decreased the GTPγS-dependent response, indicating that the same α-subunits were involved in both the hormone-dependent and -independent processes. Antibodies directed against the C-terminal region of $G\alpha_{oA}$/$G\alpha_{oB}$ (CT12) and $G\alpha_{15}/G\alpha_{16}$ (CT56) or purified preimmune serum had no inhibitory effect.

We also tried to block TRH stimulation of phosphoinositide phospholipase C activity with specific antibodies directed to internal sequences of $G\alpha_q$ or $G\alpha_{11}$ (data not shown). We could not find consistent inhibition with these antibodies, in agreement with previous work showing that internal antibodies did not inhibit other receptor-G-protein systems (Gutowsky *et al.*, 1991). The lack of effect may correlate with the fact that these antibodies do not immunoprecipitate native proteins as well as the C-terminal antibodies do.

In order to further characterize the blocking effect of the antibody, various concentrations of CT76 were incubated with the GH_3 membranes (Fig. 5*A*). Increasing amounts of antibody further decreased the PIP_2-hydrolysis; 93% reduction was achieved at 5 μM antibody and half-maximum inhibition

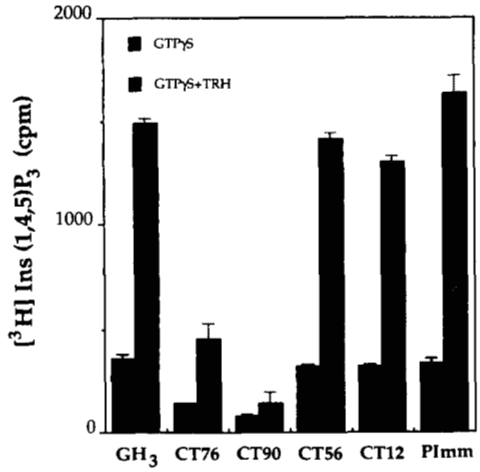

FIG. 4. **Effect of anti-Gα antibodies on PIP_2 hydrolysis in TRH-stimulated GH_3 membranes.** GH_3 membrane suspension (6 μg) was incubated for 2 h on ice with affinity purified antibody (final concentration 1.25 μM): *CT76* (Ab αq-11), *CT90* (Ab αq-11–14), *CT56* (Ab α15), *CT12* (Ab αG_{oA}) or preimmune (100 ng/ml). PIP_2 hydrolysis was performed as described under "Experimental Procedures" in the presence of 1 μM GTPγS and 100 μM TRH. The level of PIP_2 hydrolysis in control incubations (with TRH and GTPγS and without membranes) were subtracted from all values. Data shown represent the mean value of duplicated determinations. Two additional independent experiments gave similar results. *Vertical bars* denote S.E.

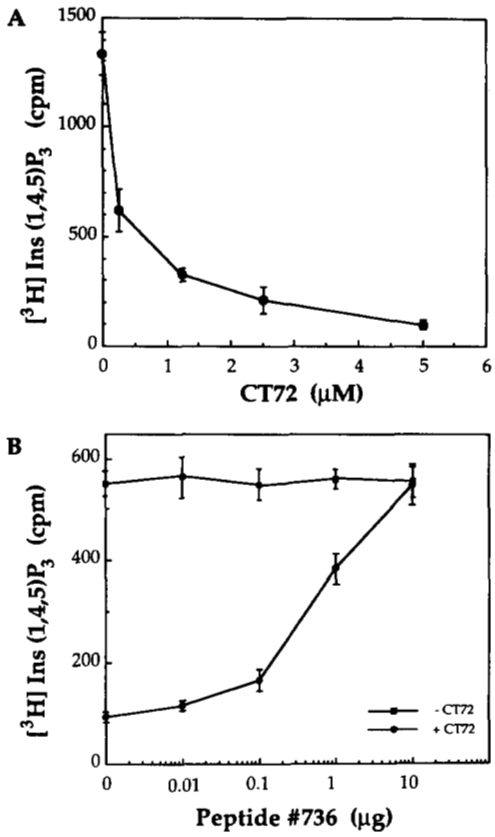

FIG. 5. **Antibody inhibition of TRH-induced PIP_2 hydrolysis in GH_3 membranes.** *A*, dependence of antibody concentration. GH_3 membranes (6 μg) were incubated for 2 h on ice with different concentrations of antibody as indicated. *B*, competition for the antibody inhibition with the peptide (no. 736, used to raise the antibody). GH_3 membrane (6 μg) suspension were incubated for 2 h on ice with different concentrations of peptide and in the presence (●) or absence (■) of $G\alpha_q$-11 antibody (5 μM). Peptide and antibody were mixed together before adding to the membranes. Samples were incubated in the presence of 1 μM GTPγS and 100 μM TRH. PIP_2 hydrolysis was performed as described under "Experimental Procedures." Data shown represent the mean values of duplicated determinations, and two additional independent experiments gave similar results. *Vertical bars* denote S.E.

was obtained with less than 0.25 μM. Addition of the peptide (used to prepare the antibody) to the reaction completely restored the PIP_2-hydrolysis (Fig. 5*B*). In contrast, the peptide had no detectable effect on membranes incubated without antibody. These results clearly demonstrate the antibody specificity for inhibiting receptor-G-protein-mediated activation of PI-PLC.

The data presented above indicate that the TRH receptor couples to the G-protein $G\alpha_q$- and/or $G\alpha_{11}$ which activate a PI-PLC enzyme leading to the induction of PIP_2 hydrolysis. Since it has been shown that $G\alpha_q$ and $G\alpha_{11}$ activate the β1 isozyme of PI-PLC (Wu *et al.*, 1992; Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Waldo *et al.*, 1991) it is possible that the TRH receptor activates the PI-PLC β1 isozyme through $G\alpha_q$/$G\alpha_{11}$ in GH_3 cells.

Reconstitution of the TRH Pathway in HEK-293 Cells—To further characterize the nature of the TRH-induced PLC activity, we have reconstituted the TRH pathway by expressing the receptor and $G\alpha_q$- or $G\alpha_{11}$-subunits in a human embryonic kidney cell line (HEK-293). Expression vectors containing the cDNAs for $G\alpha_q$- (pCIS) or $G\alpha_{11}$- (pCIS) subunits and TRH receptor (pcDNA-I) were transiently transfected into HEK-293 cells. Two days after transfection cells were harvested and the levels of G-proteins were estimated using polyclonal specific antibodies. Membranes of transiently transfected cells express 60-fold more α-subunits than non-transfected cells as estimated by densitometry of Western blots stained with $G\alpha_q$/$G\alpha_{11}$ common C-terminal antibody (Fig. 2).

Membranes of HEK-293 cells transfected with TRH receptor cDNA alone or cotransfected with $G\alpha_q$/$G\alpha_{11}$ cDNA were treated with 1 M KCl in order to wash off endogenous phospholipase C. These membranes were supplemented with purified bovine PI-PLC β1 (Ryu *et al.*, 1987) and were incubated with 1 μM GTPγS and different concentrations of the hormone in the presence of ([^{3}H]PIP_2) phospholipid vesicles. Fig. 6*A* shows that membranes isolated from cells transfected with the plasmid coding for the TRH receptor alone produce a very weak activation of PI-PLC β1. This low level of PIP_2 hydrolysis is probably due to the endogenous $G\alpha_q$ and $G\alpha_{11}$ proteins in this cell line (Fig. 2). Co-transfection of cells with $G\alpha_q$ or $G\alpha_{11}$ and TRH receptor cDNA produced a synergistic ligand-dependent increase in PIP_2 hydrolysis. Similar results were obtained with membranes prepared without washing away endogenous PLC (data not shown).

The validity of this approach was tested by transiently transfecting HEK-293 cells with cDNA encoding for the M2 acetylcholine muscarinic receptor. Expression of the M2 muscarinic receptor alone or together with either $G\alpha_q$ or $G\alpha_{11}$ showed a weak activation of phospholipase C β1 only at high concentrations of carbachol (Fig. 6*B*). This result is consistent with the observation that the activation of PIP_2 hydrolysis mediated by the M2 receptor is PTX sensitive and that it probably does not couple through G_q or G_{11} proteins (Berstein *et al.*, 1992; Ashkenazi *et al.*, 1989).

DISCUSSION

We have demonstrated the existence of two pertussis toxin-insensitive guanine-nucleotide-binding proteins α-subunits, $G\alpha_q$ and $G\alpha_{11}$, in the rat pituitary cell line GH_3. We could not detect the presence of other members of this subfamily using specific antibodies. This expression pattern is consistent with

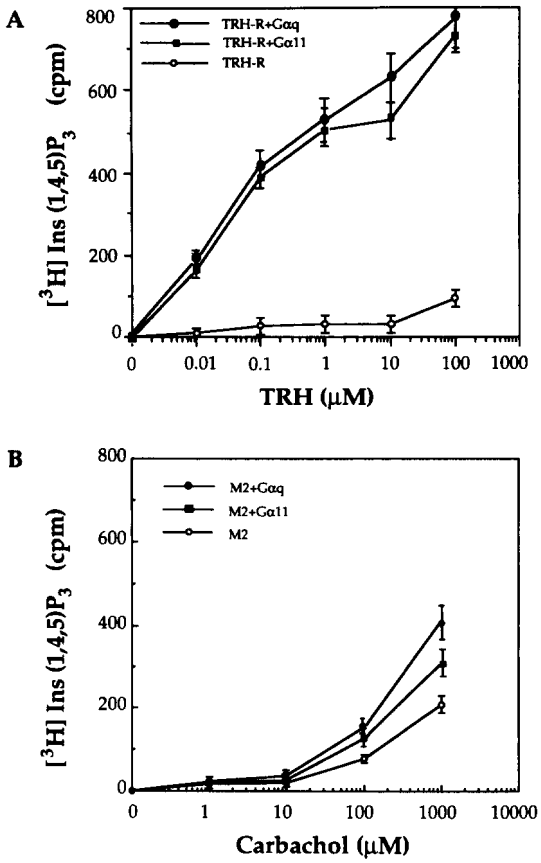

FIG. 6. **Activation of PIP_2 hydrolysis in membranes of transiently transfected HEK-293 cells.** *A*, membranes prepared from HEK-293 cells transfected with the TRH receptor cDNA alone or together with $G\alpha_q$ and $G\alpha_{11}$ cDNAs were incubated for 10 min in the presence of 1 μM GTPγS and different concentrations of TRH as indicated. *B*, membranes prepared from HEK-293 cells transfected with the M2 muscarinic receptor cDNA alone or co-transfected with $G\alpha_q$ and $G\alpha_{11}$ cDNAs were incubated for 10 min in the presence of 1 μM GTPγS and different concentrations of carbachol as indicated. Each sample contained 5 μg of membranes supplemented with 5 ng of purified PI-PLC β1 (Ryu *et al.*, 1987). Data shown represent the mean value of duplicate determinations, and two additional independent experiments gave similar results. *Vertical bars* denote S.E.

previous results indicating that $G\alpha_q$ and $G\alpha_{11}$ subunits are widely expressed in different tissues and cell lines while the $G\alpha_{15/16}$- and $G\alpha_{14}$-subunits are predominantly expressed in hematopoietic and epithelial cells, respectively (Amatruda *et al.*, 1991, Wilkie *et al.*, 1991). GH_3 cells express other $G\alpha$-subunits including $G\alpha_{oA}$ and $_B$, $G\alpha_{i\text{-}2}$, $G\alpha_{i\text{-}3}$, and $G\alpha_s$ (Paulssen *et al*, 1991). $G\alpha_{oB}$ protein apparently couples somatostatin receptor to the inhibition of calcium channels whereas the $G\alpha_{oA}$-subunit is coupled to the muscarinic receptor in GH_3 cells (Kleuss *et al.*, 1991).

TRH stimulation of prolactin release from GH_3 cells has been shown to be associated with the hydrolysis of phosphatidylinositol 4,5-bisphosphate to produce IP_3 and 1,2-diacylglycerol followed by a rapid mobilization of Ca^{2+} (Martin, 1983; Imai and Gershengorn, 1985). It has long been proposed that a pertussis-insensitive G-protein couples the TRH receptor to phosphoinositide hydrolysis (Aub *et al.*, 1986). In the present study we have shown that G-proteins of the G_q class couple the TRH receptor to a phosphoinositide phospholipase C enzyme leading to the release of inositol triphosphates. Two different antibodies which specifically recognize the C-terminal sequence of $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ subunits inhibited the hydrolysis of PIP_2 upon activation with TRH, suggesting that these α-subunits are involved in this pathway. However, since $G\alpha_{14}$ could not be detected in these cells it is likely that $G\alpha_q$ and/or $G\alpha_{11}$ are the subunits which mediated the TRH-induced activation of PIP_2 hydrolysis. It is possible that the antibodies against the carboxyl terminus are recognizing additional unknown members of this class of α-subunits. Nevertheless, the results obtained with the reconstitution of the pathway in the human kidney cell line (HEK-293) demonstrate that both proteins can couple the TRH-induced receptor to phospholipase C with similar affinities and support the idea that these α-subunits are involved in the interaction with the receptor in the rat pituitary cell line.

A similar affinity purified antibody ($G\alpha_q/\alpha_{11}$ C-terminal antibody) has been reported to attenuate the stimulation of PI-PLC by bradykinin, vasopressin, and histamine (Gutowsky *et al.*, 1991) and the activation of thromboxane-A_2 receptor in human platelets (Shenker *et al.*, 1992). Here, we have presented further evidence that supports the use of antibodies as a valuable tool in investigating the coupling of the G_q class of G-proteins in PTX-insensitive signaling pathways. We have also demonstrated that the C-terminal $G\alpha_q/G\alpha_{11}$ antibody can also recognize the $G\alpha_{14}$ subunit.

The selective inhibition of TRH induction by the antibodies indicates that the TRH receptor acts by activating one or both of the $G\alpha$-subunits: $G\alpha_q$ and/or $G\alpha_{11}$. The α-subunit of the $G\alpha_q$ and $G\alpha_{11}$ has been shown to specifically activate the phospholipase-C β1 isoform as opposed to PI-PLCγ1 or PI-PLCδ1 isoforms (Smrcka *et al.*, 1991; Taylor *et al.*, 1991; Waldo *et al.*, 1991; Wu *et al.*, 1992). Furthermore, it was found that purified $G\alpha_q$ protein could activate purified PI-PLC β1 but showed little or no activation of the β2 isoform (Park *et al.*, 1992). The reconstitution of the TRH pathway in the HEK-293 cell line has demonstrated the activation of PI-PLC β1 isoform upon stimulation by TRH through $G\alpha_q$ and $G\alpha_{11}$ subunits. These results strongly suggest that $G\alpha_q$ and/or $G\alpha_{11}$ are the subunits that couple to the TRH receptor and mediate the activation of the phospholipase C β1 isoform in the rat pituitary cell line.

Recent findings indicate that hormone-releasing factors such as TRH couple to more that one G-protein. TRH stimulates PTX-sensitive and -insensitive high affinity GTPase in membranes of GH_3 cells (Offermanns *et al.*, 1989). Thus, the $G\alpha_q$ and/or $G\alpha_{11}$ are apparently the PTX-insensitive G-proteins responsible for the TRH stimulation of inositol triphosphate release. It is still not known which PTX-sensitive G-protein mediates the stimulation of voltage-gated Ca^{2+} channels upon activation with TRH, in these cells (Gollasch *et al.*, 1991). We have found by overexpression of different α-subunits in *Xenopus* oocytes that TRH receptor can couple to the PTX-sensitive $G\alpha_{oA}$- and $G\alpha_{oB}$-subunits (in addition to $G\alpha_q$ and $G\alpha_{11}$) to increase chloride currents.[2] We suggest that these two subunits are good candidates to mediate the coupling of TRH to voltage-gated Ca^{2+} channels in GH_3 cells.

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REFERENCES

Amatruda, T. T., III, Steele, D. A., Slepak, V. Z., and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5587–5591
Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J., and Capon, D. J. (1989) *Cell* **56,** 487–493
Aub, D. L., Frey, E. A., Sekura, R. D., and Cote, T. E. (1986) *J. Biol. Chem.* **261,** 9333–9340
Aub, D. L., Gosse, M. E., and Cote, T.E. (1987) *J. Biol. Chem.* **262,** 9521–9587
Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C.,

[2] M. Quick and A. Aragay, unpublished results.

Exton, J. H., and Ross, E. M. (1992) *J. Biol. Chem.* **267,** 8081–8088
Gershengorn, M. C. (1986) *Annu. Rev. Physiol.* **48,** 515–526
Gollasch, M., Haller, H. Schultz, G., and Hescheler, J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10262–10266
Gorman, C. M., Gies, D. R., and McCray, G. (1990) *DNA Protein Engin. Tech.* **2,** 3–10.
Graham, F. L., and Van der Eb, A. J. (1973) *Virology* **52,** 456–467
Gutowski, S., Smrcka, A., Nowak, L., Wu, D., Simon, M., and Sternweis, P. C. (1991) *J. Biol. Chem.* **266,** 20519–20524
Hinkle, P. M., and Kinsella, P. A. (1984) *J. Biol. Chem.* **259,** 3445–3449
Hinkle, P. M., and Phillips, W. J. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 6183–6187
Hinkle, P. M., and Tashjian, A. H., Jr (1973) *J. Biol. Chem.* **248,** 6180–6186
Hofmann, S. L., and Majerus, P. W. (1982) *J. Biol. Chem.* **257,** 14359–14364
Imai, A., and Gershengorn, M. C. (1985) *J. Biol. Chem.* **260,** 10536–10540
Kleuss, C., Hescheler, J. Ewel, C. Rosenthal, W., Schultz, G., and Wittig, B. (1991) *Nature* **353,** 43–48
Laemmli, U. K. (1970) *Nature* **227,** 680–685
Lucas, D. O., Bajjalieh, S. M., Kowalchyk, J. A., and Martin, T. F. J. (1985) *Biochem. Biophys. Res. Commun.* **132,** 721–728
Martin, M. W., Evans, T., and Harden, T. K. (1985) *Biochem. J.* **229,** 539–544
Martin, T. F. (1983) *J. Biol. Chem.* **258,** 14816–14822
Nakamura, F., Ogata, K., Shiozaki, K., Kameyama, K., Ohara, K., Haga, T., and Nukada, T. (1991) J. *Biol. Chem.* **266,** 12676–12681
Offermanns, S., Schultz, G., and Rosenthal, W. (1989) *Eur. J. Biochem.* **180,** 283–287
Pang, I-H., and Sternweis, P. C. (1990) *J. Biol. Chem.* **265,** 18707–18712
Park, D., Jhon, D-Y., Kriz, R., Knopf, J., and Rhee, S. G. (1992) *J. Biol. Chem.* **267,** 16048–16055
Paulssen E. J., Paulssen, R. H. Haugen, T. B. Gautvik, K. M., and Gordeladze, J. O. (1991) *Mol. Cel. Endocrinol.* **76,** 45–53
Ryu, S. H., Cho, K. S., Lee, K-Y, Suh, P-G., and Rhee, S. G. (1987) J. *Biol. Chem.* **262,** 12511–12518
Shenker, A., Goldsmith, P., Unson, C. G., and Spiegel, A. M. (1991) *J. Biol. Chem.* **266,** 9309–9313
Smrcka, A. V., Hepler, J. R., Brown, K. O., and Sternweis, P. C. (1991) *Science* **251,** 804–807
Strathmann, M. P., and Simon, M. I. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9113–9117
Strathmann, M. P., and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 5582–5586
Straub, R. E., and Gershengorn, M. C. (1986) *J. Biol. Chem.* **261,** 2712–2717
Straub, R. E., Frech, G. C., Joho, R. H., and Gershengorn, M. C. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 9414–9518
Tashjian, A. H., Jr. (1979) *Methods Enzymol.* **58,** 527–535
Tashjian, A. H., Jr., Yasumura, Y., Levine, L., Sato, G. H., and Parker, M. L. (1968) *Endocrinology* **82,** 342–352
Taylor, S. J., Smith, J. A., and Exton, J. H. (1990) *J. Biol. Chem.* **265,** 17150–17156
Taylor, S. J., Chae, H. Z., Rhee, S. G., and Exton, J. H. (1991) *Nature* **350,** 516–518
Ui, M. (1990) *ADP-Ribosylating Toxins and G-proteins* (Moss, J., and Vaugham, M. eds) pp. 45–77, American Society for Microbiology, Washington, D. C.
Waldo, G. L., Boyer, J. L., Morris, A. J., and Harden, T. K. (1991) *J. Biol. Chem.* **266,** 14217–14225
Wilkie, T. M., Scherle, P. A., Strathmann, M. P., Slepak, V. Z., and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 10049–10053
Wu, D., Lee, C. H., Rhee, S. G., and Simon, M. I. (1992) *J. Biol. Chem.* **267,** 1811–1817